Deletion of the ageing gene p66Shc reduces early stroke size following ischaemia/reperfusion brain injury

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Abstract: Aims: Stroke is a leading cause of morbidity and mortality, and its incidence increases with age. Both in animals and in humans, oxidative stress appears to play an important role in ischaemic stroke, with or without reperfusion. The adaptor protein p66(Shc) is a key regulator of reactive oxygen species (ROS) production and a mediator of ischaemia/reperfusion damage in ex vivo hearts. Hence, we hypothesized that p66(Shc) may be involved in ischaemia/reperfusion brain damage. To this end, we investigated whether genetic deletion of p66(Shc) protects from ischaemia/reperfusion brain injury. Methods and results: Transient middle cerebral artery occlusion (MCAO) was performed to induce ischaemia/reperfusion brain injury in wild-type (Wt) and p66(Shc) knockout mice (p66(Shc-/-)), followed by 24 h of reperfusion. Cerebral blood flow and blood pressure measurements revealed comparable haemodynamics in both experimental groups. Neuronal nuclear antigen immunohistochemical staining showed a significantly reduced stroke size in p66(Shc-/-) when compared with Wt mice ($P < 0.05$, n = 7-8). In line with this, p66(Shc-/-) mice exhibited a less impaired neurological function and a decreased production of free radicals locally and systemically ($P < 0.05$, n = 4-5). Following MCAO, protein levels of gp91phox nicotinamide adenine dinucleotide phosphate oxidase subunit were increased in brain homogenates of Wt ($P < 0.05$, n = 4), but not of p66(Shc-/-) mice. Further, reperfusion injury in Wt mice induced p66(Shc) protein in the basilar and middle cerebral artery, but not in brain tissue, suggesting a predominant involvement of vascular p66(Shc). Conclusion: In the present study, we show that the deletion of the ageing gene p66(Shc) protects mice from ischaemia/reperfusion brain injury through a blunted production of free radicals. The ROS mediator p66(Shc) may represent a novel therapeutical target for the treatment of ischaemic stroke.

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Abstract

Background Stroke is a leading cause of morbidity and mortality, and its incidence increases with age. Both in animals and humans, oxidative stress appears to play an important role in ischemic stroke, with or without reperfusion. The adaptor protein $p66^{Shc}$ is a key regulator of reactive oxygen species (ROS) production, and a mediator of ischemia/reperfusion damage in ex-vivo hearts. Hence, we hypothesised that $p66^{Shc}$ may be involved in ischemia/reperfusion brain damage. To this end, we investigated whether genetic deletion of $p66^{Shc}$ protects from ischemia/reperfusion brain injury.

Methods and Results Transient middle cerebral artery occlusion (MCAO) was performed to induce ischemia/reperfusion brain injury in wild type (Wt) and $p66^{Shc}$ knockout mice ($p66^{Shc-/-}$), followed by 24 h of reperfusion. Cerebral blood flow (CBF) and blood pressure measurements revealed comparable hemodynamics in both experimental groups. Neuronal nuclear antigen (NeuN) immunohistochemical staining showed a significantly reduced stroke size in $p66^{Shc-/-}$ as compared to Wt mice ($P<0.05$, n=7-8). In line with this, $p66^{Shc-/-}$ mice exhibited a less impaired neurological function and a decreased production of free radicals locally and systemically ($P<0.05$, n=4-5). Following MCAO, protein levels of gp91phox NADPH oxidase subunit, was increased in brain homogenates of Wt ($P<0.05$, n=4), but not of $p66^{Shc-/-}$ mice. Further, reperfusion injury in Wt mice induced $p66^{Shc}$ protein in the basilar and middle cerebral artery, but not in brain tissue suggesting a predominant involvement of vascular $p66^{Shc}$.

Conclusion In the present study we show that deletion of the aging gene $p66^{Shc}$ protects mice from ischemia/reperfusion brain injury through a blunted production of free radicals. The ROS mediator $p66^{Shc}$ may represent a novel therapeutical target for the treatment of ischemic stroke.

Introduction

Stroke is a leading global cause of mortality, responsible for more deaths than cancer[1]. Even in patients presenting comparable degrees of ischemia, a huge variability in the recovery of brain function is observed indicating a complex pathological process which to date, still lacks a specific therapy for its effective treatment[2]. Indeed, even reopening of the stroke-related artery with either thrombolysis or catheter intervention, is still far from providing a safe and effective therapy for the majority of patients. It thus appears crucial to elucidate the molecular mechanisms underlying the pathogenesis of neuronal injury after stroke to set the basis for the design of novel effective therapeutical strategies.

Reactive oxygen species (ROS) are considered as crucial players in cerebrovascular disease[3, 4]. Several animal and human studies showed an association between ischemic stroke and increased systemic and local production of ROS[5-9]. A large body of evidence indicates nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) as a major source of ROS generation in cerebrovascular disease[3, 10]. In line with this, NADPH oxidase expression is profoundly greater in the vessel wall of cerebral rather than systemic arteries[11] and its genetic deletion in mice reduces brain infarction[12]. Recently, NADPH oxidase has been shown to be a downstream target of the adaptor protein $p66^{Shc}$ [13].

The mammalian $p66^{Shc}$ adaptor protein, together with $p46^{Shc}$ and $p52^{Shc}$, belongs to ShcA adaptor/docking protein family which plays an important role in transducing activation signals from receptors, such as growth factor, cytokines, and integrins, to downstream signaling cascades[14, 15]. Among the three isoforms, $p46^{Shc}$ and $p52^{Shc}$ are important in the regulation of growth factor induced Ras/Erk signaling[14, 15], whereas $p66^{Shc}$ is crucially involved in ROS generation and translates oxidative stress into apoptosis[14]. Genetic deletion of $p66^{Shc}$ in mice extends lifespan by 30%[16],

and slows down the progression of atherogenesis in double mutant $p66^{Shc-/-}$/$ApoE^{-/-}$ mice fed on a high fat diet[17, 18]. Deletion of $p66^{Shc}$ also protects from hyperglycemia-induced endothelial dysfunction[19, 20], reduces fat accumulation and premature death in adipose tissue [21, 22] and attenuates glomerulopathy[23, 24] in diabetic mice. The reported protective effects achieved by $p66^{Shc}$ deletion are mainly due to reduced oxidative stress, improved insulin sensitization, increased mitochondrial uncoupling and reduced triglyceride accumulation[17-19, 21-27]. In line with this, increased levels of $p66^{Shc}$ mRNA have been reported in peripheral blood mononuclear cells of type-2 diabetic patients[25], and in patients with acute myocardial infarction[28]. Enhanced expression of $p66^{Shc}$ has been reported in ethanol-induced liver damage in mice[29], chronic kidney dysfunction in spontaneous hypertension rats[30] and aged rats[30], and HIV-1 induced cell apoptosis in podocyte[31], which is associated to a lower level of ROS production and a blunted activation of NFkappaB[29, 30]. Of note, deletion of $p66^{Shc}$ was also shown to protect ex vivo perfused murine hearts from ischemia/reperfusion-induced injury[32]; however the involvement of $p66^{Shc}$ in stroke is largely unknown.

In the present study, we thus analyzed the effects of genetic deletion of $p66^{Shc}$ in ischemia/reperfusion brain injury. In particular, we investigated whether $p66^{Shc}$ knockout mice are protected from ischemia/reperfusion-induced and ROS-mediated brain injury and neurological deficits.

Material and Methods

Animal model

Experiments were performed on male, 12-14 weeks old wild type (C57Bl6J) and $p66^{Shc}$ knockout mice ($p66^{Shc-/-}$). Animals were fed on a normal chow diet and had *ad libitum* access to food and water, and were maintained at 24^{o} C under a 12 hours light/dark cycle. Study design and experimental protocols were approved by the institutional animal care committee (Licence Nr. TVA 139_2008; Kommission für Tierversuche des Kantons Zürich, Switzerland).

Middle Cerebral Artery Occlusion Model and Hemodynamics Measurements

To induce ischemia/reperfusion brain injury, a transient middle cerebral artery occlusion (MCAO) surgery was performed on both $p66^{Shc-/-}$ and wild type mice as previously described[33]. Mice were initially anesthetized with 4% of isoflurane and then maintained on 1.5% isofurane vaporized in NO_2 and O_2 (2:1). Following a midline cervical incision, the left common carotid artery (CCA), external (ECA) and internal carotid artery (ICA) were carefully exposed under an operating microscope. Thereafter, a 6-0 silicone-coated filament (Doccol Corporation, Redlands, USA) was introduced into the CCA and advanced into the ICA about 9-12 mm from the common carotid bifurcation. Rectal temperature was maintained at 37±0.5 ºC while the animals were under anesthesia through the use of circulating water pads. The thread was left in place for 60 min. After removal of the thread and wound care, animals were carefully observed and cared for and left in their cages for the next 24 hours. The same procedure was performed for sham-operated animals. However, silicone-coated filament was advanced into the ICA about 5 mm from the common carotid bifurcation, without interruption of cerebral blood flow in the middle cerebral artery.

During anesthesia, regional cerebral blood flow (rCBF) in the area of the cortex supplied by the MCA was measured using laser Doppler flowmetry (PeriFluxSystem 5000 with probe model no.418-1, Perimed AB, Järfälla, Sweden). The microtip probe was positioned and glued approximately 2 mm posterior and 6 mm lateral to bregma.

For blood pressure measurements, mice were allowed to familiarize with the procedure and equipment for a period of 1 week and then systolic (SBP) and diastolic blood pressure (DBP) and heart rate were recorded using the tail-cuff method (model LE 5002, Storage Pressure Meter, Letica, Spain).

MCAO experiments were performed blindly.

Neurological Deficit Measurement

Neurological deficit measurement was performed after 1 and 24 hours of reperfusion following MCAO, using a 4-point scale based on Bederson test[34]: Normal motor function was scored as 0, flexion of the contralateral torso and forelimb on lifting the animal by the tail as 1, circling to the contralateral side but normal posture at rest as 2, leaning to the contralateral side at rest as 3, and no spontaneous motor activity as 4. Neurological deficit measurements were performed by two people independently in a blinded way.

Stroke Size Measurement

Following 24 h of reperfusion, mice were sacrificed and perfused with PBS and relevant organs were excised. Brains were immersed in 4% formalin in phosphate buffer overnight and transferred to 30% sucrose solution for at least 3 days. Next, coronal 30-µm-thick sections were cut on a freezing microtome (Leica, Nussloch,

Germany). Stroke size was measured by immunohistochemical NeuN as previously performed (Dilution 1:100, Millipore, Temecula CA, USA)[35]. Quantification of stroke size was performed using NIH ImageJ software.

Western Blotting

Protein expression was determined by Western blot analysis. Isolated brains, basilar arteries, and middle cerebral arteries were homogenized in lysis buffer (NaCl 150 mmol/L, EDTA 1 mmol/L, NaF 1 mmol/L, DTT 1 mmol/L, aprotinin 10 µg/µl, leupeptin 10 µg/µl, Na_3VO_4 0.1 mmol/L, PMSF 1 mmol/L, and NP-40 0.5%). Protein concentration was measured according to the manufacturer's recommendations (Bio-Rad Laboratories GmbH, München, Germany). Equal amounts of protein were separated on a 10% SDS-PAGE, and transferred onto a polyvinylidene fluoride membrane (Millipore, Volketswil, Switzerland) by semidry transfer. Antibodies against gp91phox, p67phox and p47phox (all from Upstate) were used at 1:500 dilution. Anti-Shc (Cell Signaling, Danvers, MA) was used at 1:1000 dilution. Blots were normalized to α-tubulin (1:20000 dilution, Sigma), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (1:20000 dilution, Millipore, Temecula, CA). Anti-rabbit and anti-mouse secondary antibodies were purchased from GE Healthcare (Buckinghamshire, United Kingdom). For detection of protein carbonylation, dinitrophenol antibody (LifeSpan BioSciences, Inc.) at 1:200 dilution was used. All western blots were quantified by densitometric analysis performed by Scion Image Corporation software.

Measurement of ROS

O_2^- production in whole blood was determined using the spin trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (Noxygen, Germany). Blood samples were mixed with Krebs-HEPES solution containing sodium diethyldithiocarbamate trihydrate (5 µmol/L), deferoxamine methanesulfonate (25 µmol/L), and 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (200 µmol/L) (all from Noxygen, Germany) and Heparin (100 U/ml). Samples were frozen in liquid nitrogen and O_2^- production was assessed with an ESR spectrometer (Bruker, Bremen, Germany) with the following instrumental settings: center field 2.011 g, field sweep 100 G, microwave power 1 mW, modulation amplitude 10 G, sweep time 10.4 sec, number of scan 10.

Statistical analysis

Data are given as mean±SEM. Statistical analysis was done by two-way ANOVA analysis of variance with *post hoc* multiple comparisons using Bonferroni test, or paired/unpaired T-test in two way tails as appropriate. A probability value $p<0.05$ denoted a significant difference. Statistical analyses were performed using GraphPad Prism software version 4.03 (GraphPad Software Inc., La Jolla, CA, USA).

Results

Reduced brain infarction after ischemia/reperfusion injury in p66$^{Shc-/-}$ mice

To evaluate the role of p66^{Shc} in ischemia/reperfusion brain injury, a transient MCAO surgery was performed on both, p66$^{Shc-/-}$ and Wt mice, in order to induce a focal ischemic stroke. MCAO is a reliable and reproducible rodent model of cerebral ischemia in humans that has been demonstrated to result in sensorimotor and cognitive deficits[33]. Following 60 min of MCA occlusion, the silicone filament was withdrawn allowing reperfusion for 24 hrs. Following 24 hours of reperfusion both p66$^{Shc-/-}$ ($P<0.0001$) and Wt ($P=0.0086$) mice underwent a comparable weight loss (data not shown). To quantify stroke size, NeuN immunohistochemical staining was performed as previously described[35]. Morphometrical analysis revealed a significantly reduced stroke size in p66$^{Shc-/-}$ mice compared to Wt mice (Wt: 42.84±6.05 mm^2 vs. p66$^{Shc-/-}$: 16.66±7.95 mm^2; *$P=0.0196$; n=7-8; Figure 1A). Sham-operated Wt and p66$^{Shc-/-}$ did not display any lesions (data not shown).

Genetic deletion of p66^{Shc} improves neurological function following MCAO

For the assessment of neurological function after MCAO we performed a Bederson-based 4-point scale test. After 1 h of reperfusion, both, Wt and p66$^{Shc-/-}$ mice exhibited marked coordination dysfunction, shown as decreased activity, imbalance of movement, and decreased gripping ability (Wt: 1.87±0.26 vs. p66$^{Shc-/-}$: 1.43±0.17; P=N.S.; n=14; Figure 1B). Following 24 h of reperfusion p66$^{Shc-/-}$ mice showed an improved neurological function compared to Wt mice (Wt: 1.53±0.19 vs. p66$^{Shc-/-}$: 0.89±0.33; *$P=0.0142$; n= 14; Figure 1B). Moreover, neurological deficit score of p66$^{Shc-/-}$ was improved at 24 h compared to 1 h of reperfusion (24 h: 0.89±0.33 vs. 1 h: 1.43±0.17; #$P=0.0130$; n=14; Figure 1B); Such improvement was not observed in

Wt mice (24 h: 1.53±0.19 vs. 1 h: 1.87±0.26; P=0.2449; n=14; Figure 1B), suggesting an improved recovery potential in $p66^{Shc-/-}$ mice. Both Wt and $p66^{Shc-/-}$ sham operated mice, did not exhibit any neurological deficit at neither 1 nor 24 h (data not shown).

Comparable cerebral perfusion and systemic blood pressure in Wt and $p66^{Shc-/-}$ mice

Regional cerebral blood flow (rCBF) was recorded during MCAO procedure using laser Doppler flowmetry. Basal rCBF was comparable between Wt and $p66^{Shc-/-}$ mice. Following ligation of the CCA, a comparable reduction in blood flow was observed in both experimental groups (Wt: -49.6±2.69% vs. $p66^{Shc-/-}$: -51.67±9.37%; Figure 2A); similarly, after insertion of the silicon thread to achieve MCAO, a comparable degree of rCBF reduction compared to basal level was recorded in Wt and $p66^{Shc-/-}$ mice indicating a comparable degree of MCA occlusion in both groups (Wt: -84.86±2.49% vs. $p66^{Shc-/-}$: -86.13±2.77%; Figure 2A). Finally, upon retraction of the silicone thread a comparable re-establishment of blood flow was observed in both Wt and $p66^{Shc-/-}$ mice (Wt: 53.58±6.82% vs. $p66^{Shc-/-}$: 48.41±11.41%; P=N.S. for all time points; n=6-7; Figure 2A).

In order to exclude possible interference on stroke size by different systemic blood pressure in the 2 strains, blood pressure and heart rate were measured. No differences in systolic and diastolic blood pressure (SBP, DBP) as well as in heart rate were observed in both experimental groups (SBP: Wt: 154±9.866 mmHg vs. $p66^{Shc-/-}$: 146.7±8.212 mmHg; P=N.S.; Figure 2B; DBP: Wt: 124±8.083 mmHg vs. $p66^{Shc-/-}$: 128.7±7.219 mmHg; P=N.S.; Figure 2C; Heart rate: Wt: 508.3±3.930 bpm vs. $p66^{Shc-/-}$: 518.7±3.283 bpm; P=N.S.; Figure 2D).

Ischemia/reperfusion injury-induced local and systemic oxidative stress is reduced in p66$^{Shc-/-}$ mice

To address the role of oxidative stress, its level was quantified in whole brain homogenates after ischemia/reperfusion by measuring total protein carbonylation. Protein carbonylation is a widely accepted index of oxidative stress and the most common posttranslational protein modification induced by oxidative stress. Ischemia/reperfusion injury induced a significant increase in protein carbonylation in the brain of Wt stroke mice compared to that of sham operated Wt mice (Wt stroke: 207.1±40.3% vs. Wt sham: 100%; *P<0.05; n=4; Figure 3A). By contrast, no change in brain oxidative stress levels after ischemia/reperfusion was observed in p66$^{Shc-/-}$ stroke mice as compared to shams (p66$^{Shc-/-}$ stroke: 99.62±14.48% vs. p66$^{Shc-/-}$ sham: 72.77±18.17%; P=N.S.; n=3-5; Figure 3A).

To determine levels of systemic oxidative stress following ischemia/reperfusion injury, O_2^- levels were measured in whole blood using electron spin resonance spectroscopy. Wt stroke mice showed increased ROS generation after 24 h of reperfusion compared to Wt sham mice (0.068±0.009 nmol vs. 0.032±0.005 nmol; *P<0.01; n=4-6; Figure 3B). In contrast, p66$^{Shc-/-}$ stroke mice displayed comparable levels of ROS to p66$^{Shc-/-}$ sham mice (0.025±0.003 nmol vs. 0.043±0.009 nmol; P=N.S.; n=3-5; Figure 3B).

Increased protein levels of gp91phox NADPH oxidase subunit in Wt mice after MCAO

NADPH oxidase is a well known major source for ROS production in cerebrovascular disease as well as a downstream target of p66^{Shc}; to this end, its expression was measured in brain homogenates 24 h after MCAO. In the present study, protein

expression of gp91phox NADPH oxidase subunits, but not of p67phox and p47phox, was significantly increased in the brain of Wt stroke mice compared to that of Wt sham mice (gp91phox: Wt stroke: 348±69.49% vs. Wt sham: 100%; *P<0.05; n=4; Figure 4A). Interestingly, this increase was not observed in the brain of $p66^{Shc-/-}$ stroke mice compared to that of $p66^{Shc-/-}$ sham ones (gp91phox: $p66^{Shc-/-}$ stroke: 108.5±18.09% vs. $p66^{Shc-/-}$ sham: 50.14±11.73%; P=N.S.; n=3-5; Figure 4A). By contrast, p67phox (p67phox: Wt stroke: 427.1±139.1% vs. Wt sham: 100%; P=N.S.; n=3-5; Figure 4B) and p47phox (data not shown) NADPH oxidase subunit protein expression did not change in any of the experimental groups.

To exclude possible involvement of other pro- anti-oxidant enzymes we looked at protein expression of COX-2, SOD1, SOD2 and Gpx and found no significant differences in the levels of these proteins in Wt stroke versus $p66^{Shc-/-}$ stroke groups (data not shown).

Following ischemia/reperfusion $p66^{Shc}$ protein is increased in the basilar artery and middle cerebral artery but not in whole brain.

To determine regulation of $p66^{Shc}$ expression in cerebral tissues following ischemia/reperfusion, western blotting analysis was performed. $p66^{Shc}$ protein expression in the basilar artery of Wt stroke mice was profoundly increased compared to wild type sham mice (Wt stroke: 615.4±257.1% vs. Wt sham: 100±15.26%; *P=0.0245; n=7-8; Figure 5A). In line with this, $p66^{Shc}$ protein levels in the middle cerebral artery of Wt stroke mice were also elevated compared to wild type sham mice (Figure 5B). By sharp contrast, hardly any $p66^{Shc}$ expression was detected in whole brain homogenates and most importantly its levels remained unchanged after ischemia/reperfusion (Figure 5C).

Discussion

The present study demonstrates for the first time that genetic deletion of the adaptor protein $p66^{Shc-/-}$ protects mice from ischemia/reperfusion-induced brain injury and consequent neurological deficits. This effect is paralleled by a blunted activation of the pro-oxidant enzyme NADPH oxidase, a downstream target of $p66^{Shc}$, and a reduced production of free radicals.

Transient occlusion of the middle cerebral artery is a well established model of stroke[36]. Indeed, in our study, this approach led to sizable strokes and a reproducible neurological deficit. Interestingly, genetic deletion of $p66^{Shc}$ protected mice from ischemia/reperfusion-induced brain injury. Following 1 h occlusion and 24 h of reperfusion, $p66^{Shc-/-}$ displayed an over 50% reduction in cortical and subcortical brain lesions as compared to Wt mice. Our findings expand a previous report showing that $p66^{Shc}$ deletion is protective against ischemia/reperfusion injury in ex-vivo perfused hearts[32] to an in vivo setting on a different organ. Assessment of neurological deficit 1 h after MCAO denoted a similar degree of impairment in both Wt and $p66^{Shc-/-}$ mice. However, following 24 h of reperfusion $p66^{Shc-/-}$ mice displayed a marked improvement in neuromotor function compared to Wt mice. This indicates that (1) the protective effects of $p66^{Shc-/-}$ deletion interfere primarily with reperfusion injury and not with the effects of ischemia and (2) that these effects lead to a better neurological recovery after brain reperfusion. In order to exclude a possible interference by different blood pressure values and/or different degree of occlusion-reperfusion, we measured blood pressure, heart rate as well as rCBF in all experimental groups. Indeed, all hemodynamic values were comparable in Wt and $p66^{Shc-/-}$ mice indicating that the observed effect is not the result of gross physiological or procedural differences. These findings are of potential clinical importance as reperfusion injury

with subsequent brain edema is an important complication after successful thrombolysis in patients presenting with ischemic stroke[37, 38]. Nevertheless, stroke size and neurological impairment were only examined at 24 hours; although early time points are crucial, showing a preserved protective effect at later time points would be important for future developments based on the herein reported data.

Increased production of ROS is widely recognized as a key mediator of reperfusion-induced brain injury[2, 9, 39, 40], and protein carbonylation is an established marker of oxidative stress as well as the most common posttranslational protein modification induced by oxidative stress[41, 42]. Here, we report that Wt stroke mice show an increased carbonylation of brain proteins compared to Wt sham operated mice, demostrating that ischemia/reperfusion injury indeed induces oxidative stress[5-8]. In contrast, brain tissue of $p66^{Shc-/-}$ stroke mice did not display increased levels of protein carbonylation neither compared to $p66^{Shc-/-}$ shams nor to Wt shams, suggesting that $p66^{Shc}$ is crucially involved in this process. In light of the important role played by ROS in ischemia/reperfusion-induced brain injury[39, 43], strategies aimed at preventing their increase following reperfusion are currently being sought for[2, 39]. Following ischemia, increased levels of ROS promote endothelial activation and increase the permeability of brain arteries leading to the expression of adhesion molecules, proinflammatory cytokines and increased leukocyte adhesion all of which are recognized mechanisms influencing stroke size[40 39, 43]. Indeed, $p66^{Shc-/-}$ stroke mice displayed a reduced generation of ROS and a reduced stroke size. In patients with ischemic stroke increased ROS levels have been measured also in circulating blood[9]. In line with this, we found increased levels of O_2^- in peripheral blood of Wt stroke mice, but not in $p66^{Shc-/-}$ stroke mice, suggesting a systemic activation of ROS producing pathways under these conditions. Although oxidative stress is crucially

implicated in the pathogenesis of cerebrovascular disease, previous large clinical trials aimed at reducing ROS failed to improve outcome[44, 45]. Several factors could be taken into account to explain this failure: (i) High concentration of antioxidant supplements has been introduced in daily human diet due to significant improvement of healthcare (ii) endogenous antioxidant defense systems may be depressed by additional antioxidant treatment; (iii) exogenous antioxidant agent may function differently from endogenous ones; and (iv) intracellular ROS production sites may be sequestered in organelles (for instance mitochondria), thus preventing dietary antioxidants to reach their putative site of action[46]. Hence, the development of treatment strategies such as prevention of $p66^{Shc}$ activation to prevent increased ROS production, rather than lowering it, may represent a more effective alternative.

NADPH oxidase is a membrane-bound enzyme known to be expressed in cerebral arteries[47, 48] and recognized as a major source of ROS production in cerebrovascular disease[3, 10, 11]. NADPH oxidase expression is known to increase in disease conditions such as ischemia[49, 50] and its genetic deletion in mice was shown to reduce brain infarction[12]; furthermore, NADPH oxidase was recently reported to be a downstream target of $p66^{Shc}$[13]. In line with the above, protein expression of gp91phox NADPH oxidase subunit, but not of p67phox and p47phox, was strongly increased following ischemia/reperfusion in the brain of Wt stroke mice. However, this increase was not observed in $p66^{Shc-/-}$ stroke mice supporting previous reports that $p66^{Shc}$ is an upstream regulator of NADPH oxidase and that the $p66^{Shc}$/NADPH oxidase axis is crucial for ischemia/reperfusion-induced ROS[13]. To further support this associative finding, we also looked at protein expression of other anti- or pro-oxidant enzymes such as COX-2, SOD1, SOD2 and Gpx and found no significant differences in the

levels of these in Wt stroke and p66$^{Shc-/-}$ stroke groups (data not shown). Yet, the exact mechanisms linking p66^{Shc} and NAPDH oxidase deserve further investigation.

The mammalian ShcA adaptor protein is ubiquitously expressed[51-54], however, p66^{Shc} is hardly detectable in the central nervous system[55-57], with exception of the developing brain[57] and cultured rat neurons[58]. Indeed, in the present study, p66^{Shc} protein expression was hardly detectable in whole brain homogenates. Moreover, whole brain p66^{Shc} expression remained unchanged following ischemia/reperfusion suggesting a minor involvement of cerebral p66^{Shc}. By sharp contrast, we found that the basilar artery and middle cerebral artery display higher levels of basal p66^{Shc} expression as compared to whole brain. Furthermore, p66^{Shc} expression in the basilar and middle cerebral artery were dramatically increased following ischemia/reperfusion, suggesting that cerebrovascular rather than neuronal p66^{Shc} maybe an important mediator of ischemia/reperfusion brain injury. Our findings could be partially explained by previous reports indicating a pivotal role of the cerebral vasculature in determining stroke size. Indeed, following reperfusion, the endothelium of cerebral arteries is focally activated thereby promoting leukocytes leakage into the extracellular matrix and inflammation both of which are important mechanism determining neuronal damage[36, 40]. The crucial role of cerebral arteries in determining stroke size was also recently underscored by Ke-Jie *et. al.* who showed that vascular-specific deletion of PPARδ increases stroke size in the mouse via an increased post-ischemic inflammation[36]. In further support of our interpretation, is the fact that preserved endothelial function has been described as the most common mechanism protecting p66$^{Shc-/-}$ mice in several disease models including diabetes[19], atherosclerosis[18] and aging[26, 59-61] where ROS are known to play an important role.

In summary, this study shows for the first time that genetic deletion of $p66^{Shc}$ strongly reduces stroke size following ischemia/reperfusion brain injury. In line with this, $p66^{Shc-/-}$ mice displayed a far milder neurological deficit following ischemia/reperfusion as compared to Wt. The observed protective effects are likely mediated by a reduced activation of the $p66^{Shc}$ target NADPH oxidase which leads to a decreased production of free radicals. Inhibition of this novel pathway may be a novel and effective therapeutic target in preventing reperfusion injury in patients presenting with ischemic stroke undergoing thrombolysis of interventional reperfusion therapy.

Study limitations

There are some limitations that need to be acknowledged and addressed regarding the present study. First of all, the use of knock out animals does not completely exclude the possibility of some adaptive mechanisms of compensatory nature taking place over the course of their life. Secondly, the observed blunted activation of NAPDH subunits observed in $p66^{Shc-/-}$ stroke mice, needs to be investigated further to elucidate the pathways involved. Lastly, in order to fully support our conclusions with respect to possible clinical applications, future studies including later time points as well as larger animal models and human proof-of-principle experiments should be conducted.

Perspectives

Over the last century, an impressive increase in human life expectancy occurred, hence, also due to constant birth rates, the population is aging. With ascending age, the incidence of cerebrovascular diseases, such as stroke, sharply increases. Although stroke is a leading cause of morbidity and mortality to date, no effective therapeutical strategy exists. In the present study, we have shown for the first time that genetic deletion of $p66^{Shc}$ protects mice from ischemia/reperfusion brain injury through a blunted activation of NADPH oxidase and a reduced production of free radicals. Hence, $p66^{Shc}$ represents an interesting novel target to be investigated in the context of ischemic stroke and reperfusion injury.

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Conflict of Interest

None.

Figure Legends

Figure 1. Stroke size and neurological deficit measurement after ischemia/reperfusion brain injury. A, Representative NeuN immunohistochemical staining 24 h after MCAO in Wt and $p66^{Shc-/-}$ mice. Arrows indicate subcortical and cortical infarct components. $p66^{Shc-/-}$ mice show reduced stroke size compared to Wt mice, *P= 0.0196. Values are given as mm^3. B, Bederson test. Neurological deficit was measured either at 1 h of reperfusion or at 24 h of reperfusion. $p66^{Shc-/-}$ stroke mice show improved neurological function at 24 h of reperfusion compared to Wt stroke mice and to $p66^{Shc-/-}$ stroke mice at 1 h of reperfusion. *P=0.0142 for Wt stroke (24 h) vs. $p66^{Shc-/-}$ stroke mice (24 h). #P=0.0130 for $p66^{Shc-/-}$ stroke (24 h) vs. $p66^{Shc-/-}$ stroke (1 h).

Figure 2. Cerebral blood flow and arterial blood pressure measurements. A, Laser Doppler measurements revealed no difference in regional cerebral blood flow pre-MCAO between wild type and $p66^{Shc-/-}$ mice and show a similar alteration upon MCA occlusion and reperfusion. B,C,D, Wild type and $p66^{Shc-/-}$ mice do not differ in systolic and diastolic blood pressure (SBP, DBP) as well as in heart rate.

Figure 3. Production of free radicals locally (brain) and systemically (in whole blood). A, Wt mice, but not $p66^{Shc-/-}$ mice, show increased brain protein carbonylation after ischemia/reperfusion brain injury. Values are given as % of Wt sham operated mice. * P<0.05 for Wt stroke vs. Wt sham. B, Electron spin resonance spectroscopy measured increased O_2^- levels in whole blood of Wt stroke mice but not in $p66^{Shc-/-}$. Values are given as nmol O_2^-. * P<0.05 for Wt stroke vs. Wt sham.

Figure 4. Protein levels of NADPH oxidase subunits in whole brain homogenates. Western blotting reveals increased protein expression of gp91phox NADPH oxidase

subunit in brain homogenates of Wt stroke mice, but not of $p66^{Shc-/-}$ (A). In contrast, p67phox NADPH oxidase subunit expression does not change in any of the experimental groups (B). Values are given as % of Wt sham. * $P<0.05$ for Wt stroke vs. Wt sham.

Figure 5. $p66^{Shc}$ protein levels in basilar artery, middle cerebral artery and brain. A,B, $p66^{Shc}$ protein expression is increased in basilar artery and middle cerebral artery of Wt stroke mice compared to Wt sham mice. C, Protein level of $p66^{Shc}$ in whole brain homogenates is hardly detectable and remains comparable between Wt stroke and Wt sham mice. Values are given as % of Wt sham mice. * $P=0.0245$ for Wt stroke vs. Wt sham.

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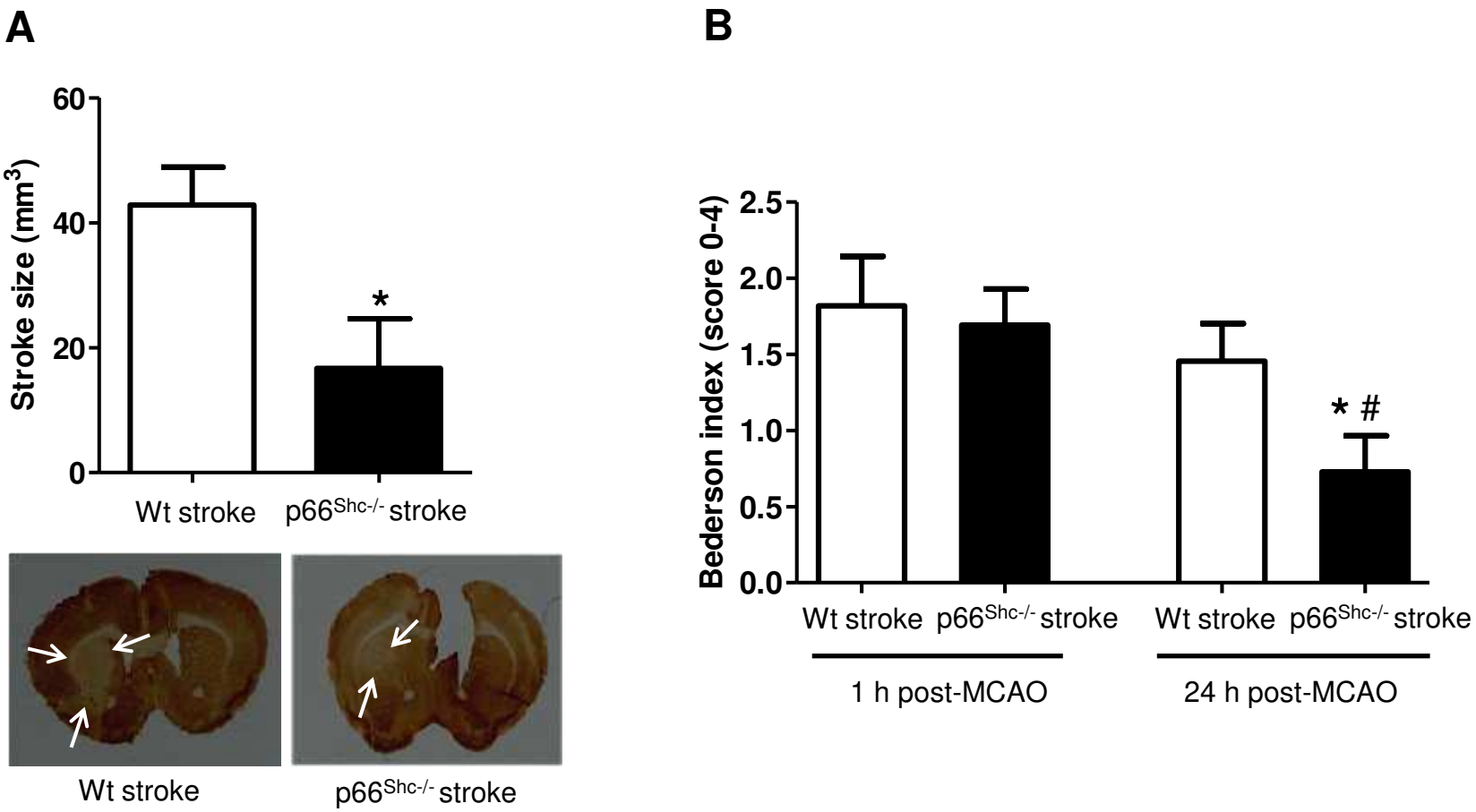
A
60
40
20
0
Stroke size (mm3)
*
Wt stroke
p66Shc-/- stroke
Wt stroke
p66Shc-/- stroke
B
2.5
2.0
1.5
1.0
0.5
0.0
Bederson index (score 0-4)
* #
Wt stroke
p66Shc-/- stroke
Wt stroke
p66Shc-/- stroke
1 h post-MCAO
24 h post-MCAO

Figure 1

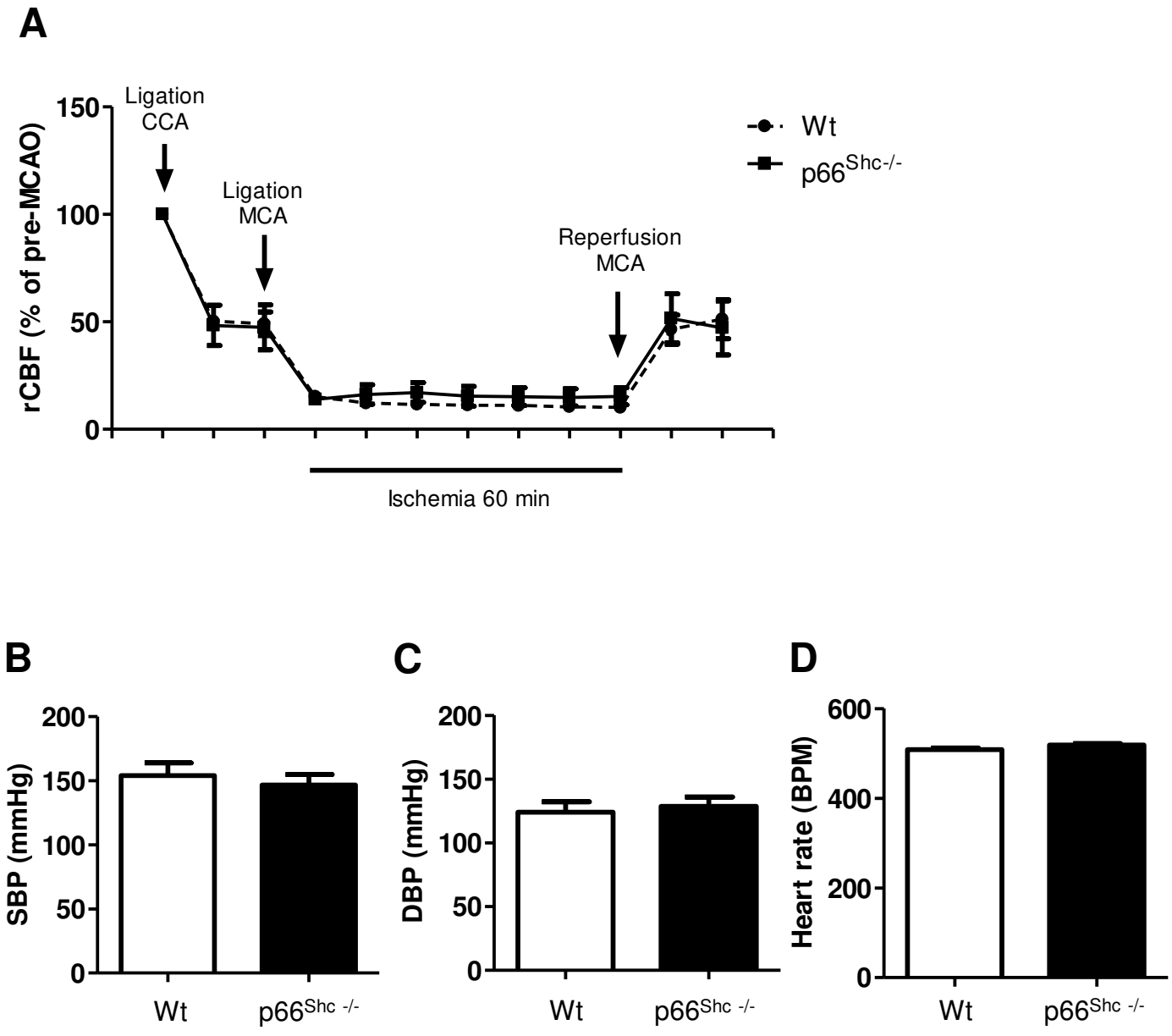
A
Ligation
CCA
Ligation
MCA
Reperfusion
MCA
Wt
p66Shc-/-
rCBF (% of pre-MCAO)
150
100
50
0
Ischemia 60 min
B
SBP (mmHg)
200
150
100
50
0
Wt
p66Shc -/-
C
DBP (mmHg)
200
150
100
50
0
Wt
p66Shc -/-
D
Heart rate (BPM)
600
400
200
0
Wt
p66Shc -/-

Figure 2

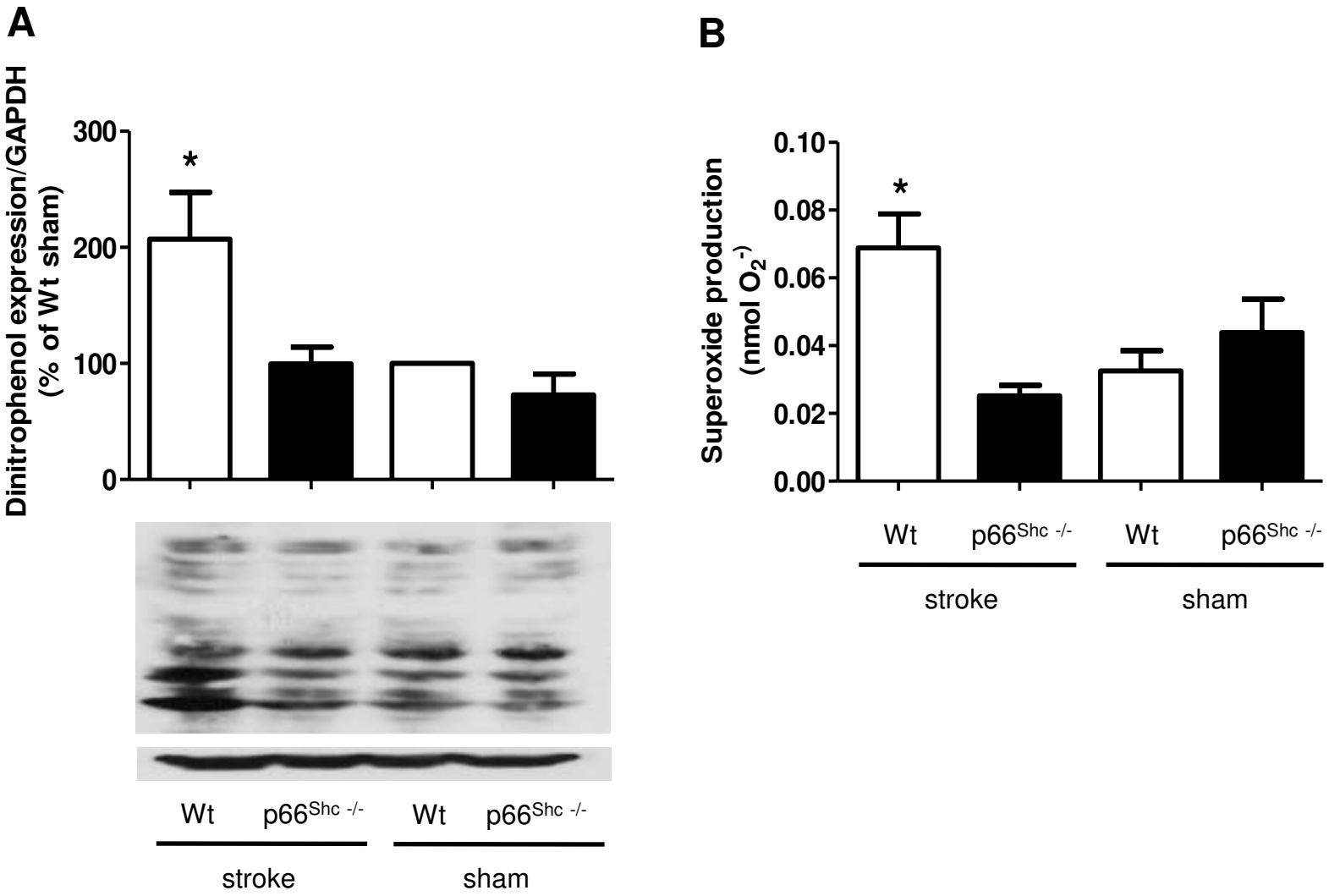
A
Dinitrophenol expression/GAPDH
(% of Wt sham)
300
200
100
0
*
Wt
p66Shc -/-
Wt
p66Shc -/-
stroke
sham
B
Superoxide production
(nmol O2-)
0.10
0.08
0.06
0.04
0.02
0.00
*
Wt
p66Shc -/-
Wt
p66Shc -/-
stroke
sham

Figure 3

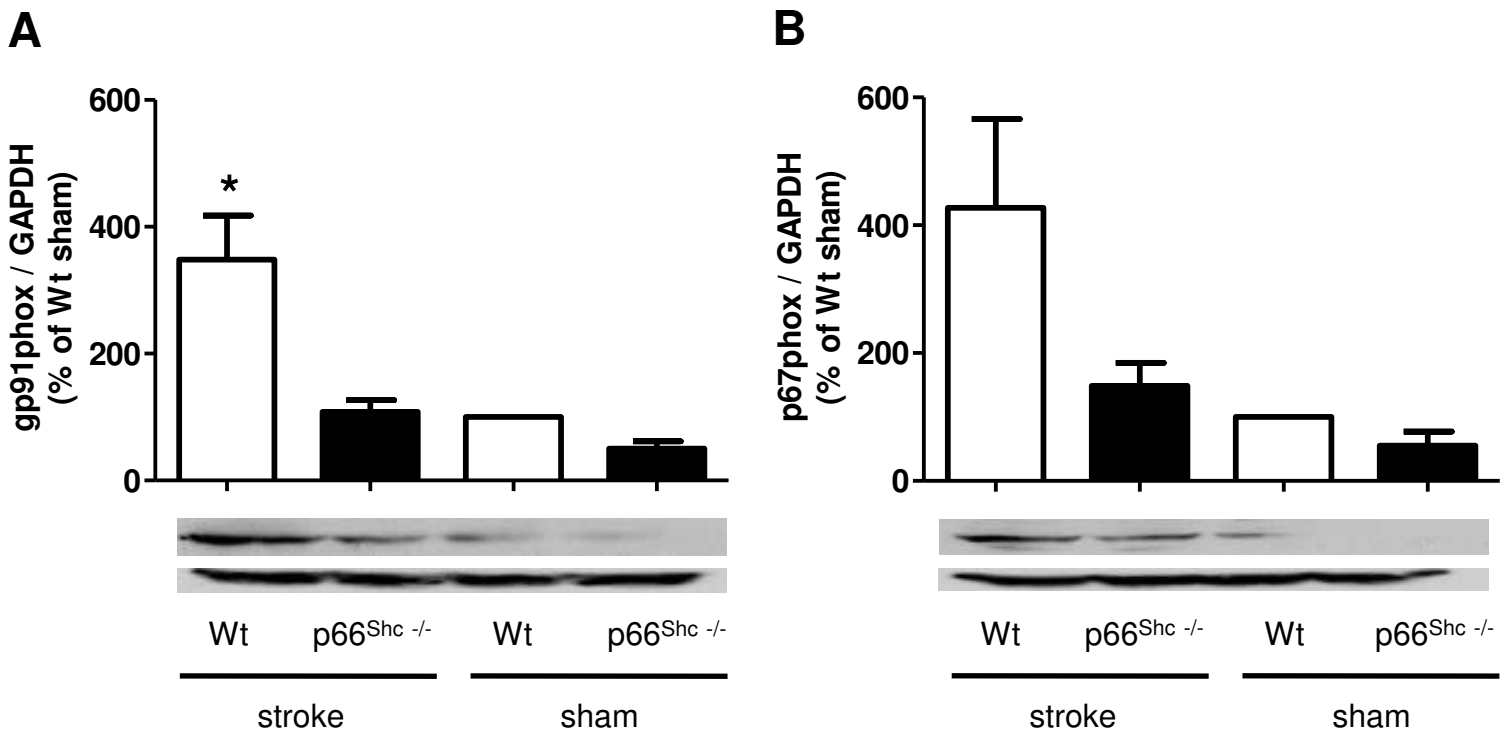
A
gp91phox / GAPDH
(% of Wt sham)
600
400
200
0
*
Wt
p66Shc -/-
Wt
p66Shc -/-
stroke
sham
B
p67phox / GAPDH
(% of Wt sham)
600
400
200
0
Wt
p66Shc -/-
Wt
p66Shc -/-
stroke
sham

Figure 4

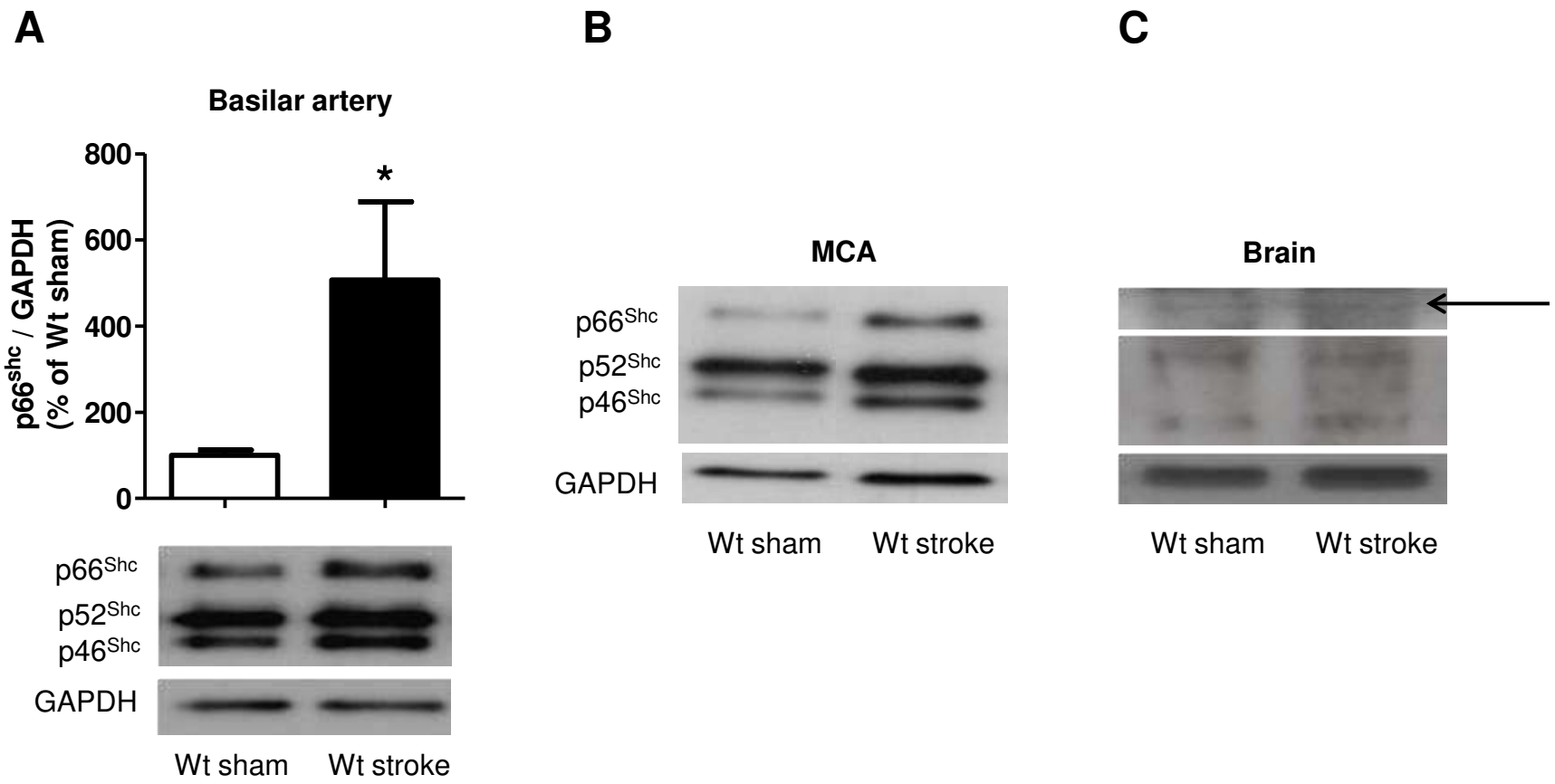
A
Basilar artery
800
600
400
200
0
p66shc / GAPDH
(% of Wt sham)
*
p66Shc
p52Shc
p46Shc
GAPDH
Wt sham
Wt stroke
B
MCA
p66Shc
p52Shc
p46Shc
GAPDH
Wt sham
Wt stroke
C
Brain
Wt sham
Wt stroke

Figure 5